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John Lee

Attorney Docket No.: 13353.1073  
PATENT APPLICATION

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

In re application of **Matthew Glenn, Ilkka Havukkala, Mark William Lubbers,  
and James Dekker**

Group Art Unit: 1652

Application No. : 10/650,274  
Filed : August 28, 2003  
For : **POLYNUCLEOTIDES AND POLYPEPTIDES, MATERIALS  
INCORPORATING THEM AND METHODS FOR USING THEM**  
Examiner : Malgorzata Walicka

**DECLARATION OF DR. JAMES DEKKER**

MS: AMENDMENT  
Commissioner for Patents  
P. O. Box 1450  
Alexandria, VA 22313-1450

The undersigned, Dr. James Dekker, hereby declares:

1. I am presently a Senior Research Scientist at Fonterra Co-Operative Group, the assignee of the subject patent application, and an inventor of the claimed subject matter. I have a PhD in the field of molecular biology and immunology. The following studies were carried out under my supervision.

2. Pyruvate oxidase (EC 1.2.3.3) acts as an oxoreductase with activity towards pyruvate and other  $\alpha$ -keto acids. It requires thiamine diphosphate and flavin adenine dinucleotide (FAD) as co-factors, and also exhibits decarboxylase activity as it catalyses the release of carbon dioxide (CO<sub>2</sub>) from the  $\alpha$ -keto acid pyruvate.

Comparison of predicted amino sequences of the pyruvate oxidase enzyme from *Lactobacillus plantarum* (POW) and AP4 of *L. rhamnosus* HN001 (SEQ ID NO: 172 of the instant specification) showed that most of the amino acids important for co-factor binding and enzyme activity in *L. plantarum* pyruvate oxidase were present in the AP4 amino acid sequence, indicating that the AP4 nucleotide sequence of SEQ ID NO: 73 encoded a pyruvate oxidase (Exhibit 1).

More specifically, Exhibit 1 shows an alignment of predicted amino acid sequences of pyruvate oxidase genes from *Lactobacillus plantarum* (GenBank accession no. 1POW\_B; 1POW) and *Lactobacillus rhamnosus* HN001 SEQ ID NO: 172 (HN001\_AP4). Identical amino acids at each position are shaded. Most of the amino acids found to be important for 1POW substrate binding and enzyme activity according to X-Ray crystallographic studies (Muller YA, Schumacher G, Rudolph R, Schulz GE; *Jnl. Mol. Biol.* 237:315-335, 1994) are preserved in AP4. Symbols under the aligned amino acid sequences indicate 1POW amino acids found to be important for glycerol binding ( $\Delta$ ), thiamine diphosphate binding ( $\cdot$ ), FAD binding ( $\blacktriangle$ ), enzyme active site ( $\clubsuit$ ), and metal ion binding ( $\circ$ ). Dotted line above the alignment indicates the presence in both sequences of Prosite motif PS00187, a signature of thiamine pyrophosphate enzymes ([LIVMF]-[GSA]-x(5)-P-x(4)-[LIVMFYW]-x-[LIVMF]-x-G-D-[GSA]-[GSAC]).

To determine whether the enzyme encoded by the AP4 nucleotide sequence exhibited decarboxylase activity toward  $\alpha$ -keto acid substrates, the full-length polynucleotide sequence of pyruvate oxidase AP4 from *L. rhamnosus* strain HN001, given in SEQ ID NO: 73 was amplified from *L. rhamnosus* HN001 DNA using standard PCR methodology. AP4 PCR product was then cloned into pFRC019, an in-house expression vector, and transformed into *L. rhamnosus* HN001.

The  $\alpha$ -keto acid decarboxylating activity of AP4 was tested using three substrates,  $\alpha$ -ketoisocaproate,  $\alpha$ -ketomethylvalerate, and  $\alpha$ -ketoisovalerate that yield 3-methylbutanal (3MB), 2-methylbutanal (2MB), and 2-methylpropanal (2MP), respectively, following  $\alpha$ -keto acid decarboxylation. Cell-free extracts were prepared from *L. rhamnosus* HN001 carrying the AP4 genetic construct, *L. rhamnosus* HN001 carrying empty pFRC019 expression plasmid vector, *L. rhamnosus* HN001 carrying a genetic construct for an irrelevant protein, and a *Lactococcus lactis* strain B1113

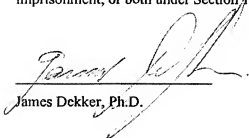
known to exert strong  $\alpha$ -keto acid decarboxylating activity as a positive control. In all cases, cell extracts were prepared from late log-phase cells grown in M17 media using a French pressure cell at 18000 psi, and the supernatants filtered through a 0.45  $\mu$ m hydrophilic membranes (Millipore). A time-course assay of  $\alpha$ -keto acid decarboxylating activity was performed at 30 °C, pH 6.3, using 20 mM of the different  $\alpha$ -keto acid substrates and other reaction conditions as described by de la Plaza et al. (*FEMS Microbiol. Lett.* 238:367-74, 2004). As an internal standard, *Lactococcus lactis* MG5295 extracts were spiked with 20–100  $\mu$ M of the expected reaction products. Enzyme activity was assayed over a 6 h period by fast GCMS headspace analysis in 1.5 ml vials using set ion m/z ratios of 58 for 3-methylbutanal, 57 for 2-methylbutanal and 72 for 2-methylpropanal. Peak height data from chromatograms were plotted vs. time and used to calculate enzyme activities as nmol/min/mg total protein (Table 1).

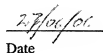
**Table 1.** Decarboxylase enzyme activity on three  $\alpha$ -keto acid substrates. Numbers show enzyme activity as production of each reaction product (nmol/min/mg total protein) assayed over 6 h.

Cell extract	3-Methylbutanal	2-Methylbutanal	2-Methylpropanal
<i>L. rhamnosus</i> HN001 + vector only	0.002	0.002	0.002
<i>L. rhamnosus</i> HN001 + irrelevant protein	0.001	0.002	0.003
<i>L. rhamnosus</i> HN001 + AP4	0.028	0.048	0.044
<i>L. lactis</i> B1113	0.188	0.137	0.496

As shown in Table 1, while cell extracts of *L. rhamnosus* HN001 carrying empty expression vector or expression vector encoding an irrelevant protein showed no significant decarboxylation of the  $\alpha$ -keto acid substrates, the cell extract of *L. rhamnosus* HN001 containing the expression vector encoding pyruvate oxidase AP4 showed significant enzyme activity toward all three substrates. Therefore, based on both amino acid comparison and enzyme activity assays, the *L. rhamnosus* HN001 gene AP4 (SEQ ID NO: 73) encodes pyruvate oxidase.

3. The undersigned further declares that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and further that these statements were made with the knowledge that willful, false statements, and the like so made are punishable by fine or imprisonment, or both under Section 1001 of Title 35 of the United States Code.

  
James Dekker, Ph.D.

  
Date

# Exhibit 1

1POW	1	MVMQTKQTN	ILAGAAVIKV	LEAWGVHDLY	GIPGGSINSI	MDALSAERDR	50
HN001_AP4		.MEASVMTEK	INAADAMIKV	LEDWGIHNIY	GLPGGSFDST	MNALYNRRHT	
					ΔΔ		
1POW	51	IHYIQVRHEE	VGAMAAAADA	KLTKGIGVCF	GSAGPGGTHL	MNGLYDAREH	100
HN001_AP4		INYVQVRHEE	VGALAAAGEA	KVTGRIGATF	GSAGPGVAHL	LNGLYDAQYD	
		**			*	*	▲▲
1POW	101	HVPVLALIGQ	FGTGMNMDT	FOEMNENPIY	ADVADYNVTA	VNAATLPHVI	150
HN001_AP4		HVPVLALVGO	VPTEAMNTNY	FOEMNENPMF	ADVSVYNRTA	MTAAQLPHVV	
		▲		♦♦			
1POW	151	DEAIRRAYAH	QGVAVVQIEV	DLPWQOIPAE	DWYASANSYQ	TPLLPEPDVQ	200
HN001_AP4		DEAIRQAYKY	QGVAVVTIEK	DLGWQEI.DD	NYVSSANLYQ	KPLLPEPDPE	
1POW	201	AVTRLTQTLL	AAERPLIYYG	IGARKAGKEL	EQLSKTLKIP	LMSTYPARKI	250
HN001_AP4		QVATAWSILK	DAKKPILYVG	NGARGARDEI	IAFSEKTHIP	IITITALAKGV	
			▲	▲		▲▲▲	
1POW	251	VADRYPAYLG	SANRVAQKPA	NEALAQADV	LFVGNNYPFA	EVSKAFKNTR	300
HN001_AP4		VPDDYKANMG	SAGRVASKEG	VEVARGADTV	LFLGSDFFPQ	PFFIAPN..A	
			▲♦♦		▲▲♦		
1POW	301	YFLQIDIDPA	KLGRHKHTDI	AVLADAQKTL	AAILAQVSER	ESTPNWQANL	350
HN001_AP4		KYIQVDIDAS	KFGRRHTVDL	AVLADAKKFI	KALTEKADAV	PETAWYRAAV	
		▲▲	▲	▲▲			
1POW	351	ANVKNNRAYL	ASLEDKQEGP	LQAYQVLRAV	NKIAEPDAIY	SIDVGDINLN	400
HN001_AP4		ANKANNAEWM	TSFEDDSQTP	LRVEPIFKLI	NEMADKDAVF	QVDVGNVTIN	
						* **	
1POW	401	ANRHLKLTPS	NRHITSNLFA	TMGVGIPGAI	AAKLNYPERQ	VFNLAGDGGA	450
HN001_AP4		GMRYLKANDN	QIFTTSGWYA	TMGYAVPAAI	GAQAEPDRQ	VWSISGDGGF	
			▲ *	*		♦♦*	
						*	
1POW	451	SMTMQDLATQ	VQYHLEVINV	VFTNCQYGF	KDEQEDTNQN	DFIGVEFNDI	500
HN001_AP4		AMVMQDIMTQ	VKYHMPPIINI	VLTNESLGF	EAEQDDTRQ	.HSGVDLIDA	
		◊ ◊		* ◊ * ♦♦*	Δ		
				*	*		
1POW	501	DFSKIADGVH	MQAFRVNKIE	QLPDVFEQAK	AIAQHEPVLI	DAVITGDRPL	550
HN001_AP4		DYGAKEAEAMG	AQGFEVHNLD	ELKAARAKAK	..DRKGPEVVI	DVKISDLRPI	
1POW	551	PAEKLRLDSA	MSSAADIEAF	KORYEAQDLQ	PLSTYLKQFG	LDD....	597
HN001_AP4		PVEQLVLDKQ	TQDPEAVDAF	VKKYHAETLI	PFGSYSRMLK	RQLQTFN	